

Interleukin 6 Is Essential for In Vivo Development of B Lineage Neoplasms

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Summary

Interleukin (IL) 6 has been suggested to be the major cytokine responsible for proliferation of neoplastic plasma cells in both human myeloma and mouse plasmacytoma. Much of the evidence supporting this suggestion is derived from in vitro studies in which the survival or proliferation of some plasma cell tumors has been found to be IL-6 dependent. However, it remains unclear whether this dependency is the consequence of in vivo or in vitro selective pressures that preferentially expand IL-6-responsive tumor cells, or whether it reflects a critical in vivo role for IL-6 in plasma cell neoplasia. To address this question, we have attempted to induce plasma cell tumors in normal mice and in IL-6-deficient mice generated by introduction of a germline-encoded null mutation in the IL-6 gene. The results demonstrate that mice homozygous (+/+) or heterozygous (+/-) for the wild-type IL-6 allele yield the expected incidences of plasma cell tumors. In contrast, mice homozygous for the IL-6-null allele (-/-) are completely resistant to plasma cell tumor development. These studies define the essential role of IL-6 in the development of B lineage tumors in vivo and provide experimental support for continued efforts to modulate this cytokine in the treatment of appropriate human B cell malignancies.

IL-6 is a pleiotropic cytokine associated with a diverse set of systemic and tissue-specific biological responses (for review see references 1-4). Many of these responses occur within the immune system, where the effects of IL-6 include enhancement of hematopoietic progenitor cell proliferation (5), initiation of primary immune responses (6), induction of terminal B cell differentiation (7, 8), and activation of T cells (9-13). In addition to mediating these normal immune functions, IL-6 is also a growth factor for several neoplastic cell types. In mice, IL-6 is thought to be important for the growth of plasmacytomas arising in the granulomatous tissue formed by the intraperitoneal injection of pristane. The evidence supporting this conclusion is twofold. First, the in vitro proliferation of plasmacytomas derived from primary tumor isolates (14) as well as some established plasmacytoma cell lines is enhanced or completely dependent on addition of IL-6 to the medium (14-19). Second, treatment of mice with anti-IL-6 or anti-IL-6R antibody protects ~50% of the animals from subsequent challenge with an in vitro-cultured IL-6-dependent plasmacytoma cell line (17).

The apparent role of IL-6 in plasma cell neoplasia is fur-

ther supported by studies of an analogous human disease, multiple myeloma. Like the murine granuloma, the bone marrow microenvironment in which malignant plasma cells develop is a rich source of IL-6. Recent studies indicate that the levels of this cytokine are routinely elevated in myeloma patients (18-20), possibly through direct interaction of tumor cells with bone marrow-derived stromal elements (21). Although the functional relevance of elevated IL-6 levels in these patients remains unclear, the demonstrated ability of primary myeloma explants (22, 23) and some myeloma-derived cell lines (24-27) to proliferate in response to IL-6 suggests that this cytokine may be an important myeloma growth factor. Consistent with this view is a recent study in which anti-IL-6 or anti-IL-6R antibodies have transiently reduced tumor burden in a late-stage myeloma patient (28).

The murine and human studies described above clearly demonstrate that the proliferation of some neoplastic plasma cells is IL-6 dependent. More recent studies have demonstrated that a subset of these tumors is also induced to proliferate in response to other cytokines such as oncostatin M, ciliary neurotropic factor, IL-11, and leukemia inhibitory factor (4, 29, 30), whose cell surface receptors use a common gp130 signal-transducing molecule. In light of this functional redundancy among cytokines, it remains unclear whether the IL-6 dependency of plasma cell tumors is the consequence of subtle

†This manuscript is dedicated to the memory of Georges Köhler, whose untimely death occurred during the course of these studies.

in vivo or in vitro selective pressures that preferentially expand IL-6-responsive tumor populations, or whether IL-6 dependency truly reflects a unique and requisite role for IL-6 during the development of plasma cell tumors. In the current study, we have addressed these possibilities by attempting to induce plasma cell tumors in mice made deficient in IL-6 production by a targeted germline-null mutation in exon 2 of the IL-6 gene (31). The induction protocol chosen uses the *myc/raf*-expressing retrovirus, J3V1, that has previously been shown to induce both myeloid and plasma cell tumors in normal BALB/c mice (32–35) and B cell lymphomas in T cell-deficient nude mice (35). If IL-6 dependency is a consequence of preferential expansion of IL-6-responsive tumor cells, IL-6-deficient mice should yield B or plasma cell tumors that will be IL-6 independent but may display novel cytokine requirements. If, on the other hand, IL-6 imparts a unique and requisite signal during tumorigenesis, IL-6-deficient mice should be resistant to tumor development.

Materials and Methods

Mice. Mice heterozygous for a germline-encoded null mutation in exon 2 of the IL-6 gene (31) were backcrossed to plasmacytoma-susceptible BALB/cAnNCR mice (Frederick Cancer Research Facility, Frederick, MD) for three generations using an accelerated breeding scheme in which successive generations were derived from progeny containing the greatest proportion of BALB/c chromosomes as determined by simple sequence length polymorphism analysis of chromosome-specific markers that distinguish BALB/c, C57BL, and 129 inbred strains. Resultant N3 mice were interbred to yield homozygous IL-6-deficient (–/–), heterozygous (+/–), and homozygous IL-6 wild-type (+/+) animals. N3F1 homozygotes were further interbred to generate sufficient numbers of mice for use in induction studies. All mice were housed in the specific pathogen-free National Cancer Institute animal facility (Frederick, MD) under barrier conditions.

Tumor Inductions. Tumors were induced in mice by a single intraperitoneal injection of 0.5 ml pristane (Sigma Chemical Co., St. Louis, MO) 2 d before intraperitoneal injection of 1.25×10^4 focus-forming units (0.2 ml) of the *myc/raf*-containing J3V1 retrovirus (32, 34, 35). Tumor-bearing mice were killed and ascites fluid or tumor-bearing granulomatous tissue was transferred to pristane-primed BALB/c mice for subsequent tumor analyses.

PCR-based Identification of IL-6-deficient Allele. The presence of the defective IL-6 allele in mice and tumor samples was determined by PCR as previously described (31). Briefly, three PCR primers were used to identify the wild-type and null alleles: P1 (TTCCATC-CAGTTGCCTTCTTGG), an upstream primer hybridizing to the 5' portion of exon 2 in the IL-6 locus; P2 (TTCTCATTTCCACATTTCCCAG), a downstream primer hybridizing to the 3' portion of exon 2 in the IL-6 locus; and Pneo (CCGGAGAACCTG-CGTGCAATCC), a downstream primer hybridizing to sequences within the neomycin gene used to disrupt exon 2. Using P1 and P2, the wild-type allele is amplified as a 174-bp fragment while the mutated allele is 1,314 bp. Although these fragments are sufficient to differentiate wild-type and mutant alleles, the preferential amplification of the smaller wild-type allele makes identification of heterozygotes (+/–) difficult. Accordingly, an additional PCR reaction was performed using P1 and Pneo that selectively amplifies the mutant allele as a 380-bp fragment.

Flow Cytometric Analyses. The cell surface phenotype of normal

and tumor cell populations was determined by flow cytometric analyses as previously described (34, 35). Tumor samples were analyzed using the following reagents, all of which were purchased from Pharmingen (San Diego, CA): FITC- or PE-rat anti-mouse CD45, FITC-rat anti-mouse ThB, PE-rat anti-mouse CD45 (B220), PE-anti-mouse CD11b, FITC-anti-mouse Ia^d, PE-CD43, PE-GR-1, and FITC-anti-mouse CD5. The specificity of each reagent used in these analyses was confirmed on normal mouse spleen, lymph node, or appropriate tumor cell lines. FITC- and PE-labeled irrelevant antibodies were included in each assay as controls for nonspecific staining. FcR-mediated binding of labeled antibodies was precluded by preincubation of cells with unlabeled mAb 2.4G2 specific for mouse FcγRII (36). All samples were analyzed on a FACScan® (Becton Dickinson and Co., San Jose, CA). Nonviable cells were excluded by propidium iodide staining.

In Situ Hybridization. Freshly isolated tumor tissues were fixed in 10% buffered formalin, paraffin embedded, sectioned, and hybridized to IgL Cκ- and λ-specific probes as described (Hausner, P., and S. Rudikoff, manuscript in preparation).

Results and Discussion

To assess directly the in vivo role of IL-6 in the development of plasma cell tumors, we have compared the incidence, latency, and phenotype of J3V1-induced tumors arising in mice homozygous for a germline-null mutation in the IL-6 gene (–/–) with those developing in heterozygous (+/–) and homozygous wild-type (+/+) animals. In a preliminary induction study, it was determined that +/- mice derived from the original 129 × C57Bl/6 chimeric stock failed to yield plasma cell tumors (data not shown). These results suggested that the genetic background of the chimeric animals, like that of most other inbred mouse strains, was resistant to plasma cell tumor development. To generate susceptible, IL-6-deficient mice, the IL-6-null allele was backcrossed onto the plasmacytoma-susceptible BALB/c background for three generations. The resultant mice (+/+, +/-, and –/–) were subjected to the pristane/J3V1 induction protocol, and the phenotypes of arising tumors were compared with those developing in similarly treated BALB/c mice.

The hematopoietic lineage association of tumors arising in all animals was determined by a combination of flow cytometric and immunohistologic analyses. As shown in Fig. 1 A, a typical plasma cell tumor expresses membrane-associated ThB but not the B lymphoid marker CD45R (B220), the myeloid lineage marker CD11b, or MHC class II determinants. Minor populations of CD11b⁺ cells are also found in the ascites of virtually all tumor-bearing mice and represent normal MHC class II positive and negative peritoneal macrophages. In situ hybridization with L chain-specific probes indicates that plasma cell tumors also express high levels of cytoplasmic IgL-encoding messenger RNA (Fig. 1 A, right). The H chain expressed in these tumors is predominantly IgM with an occasional IgA- or IgG-producer detected (data not shown). In contrast, myeloid tumors are readily identified by their expression of membrane-associated CD11b but not ThB, CD45R(B220), or cytoplasmic L chain (Fig. 1 B). MHC class II expression on myeloid tumors is variable, with approximately half of all myeloid tumors expressing these determinants

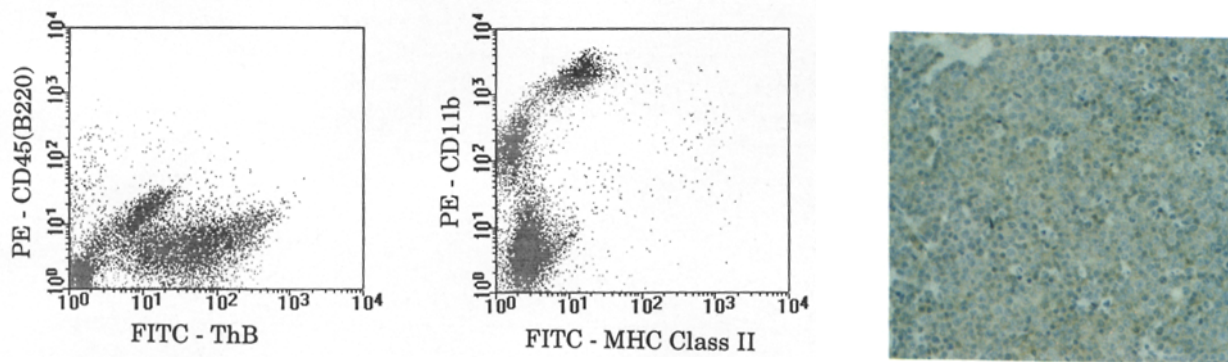
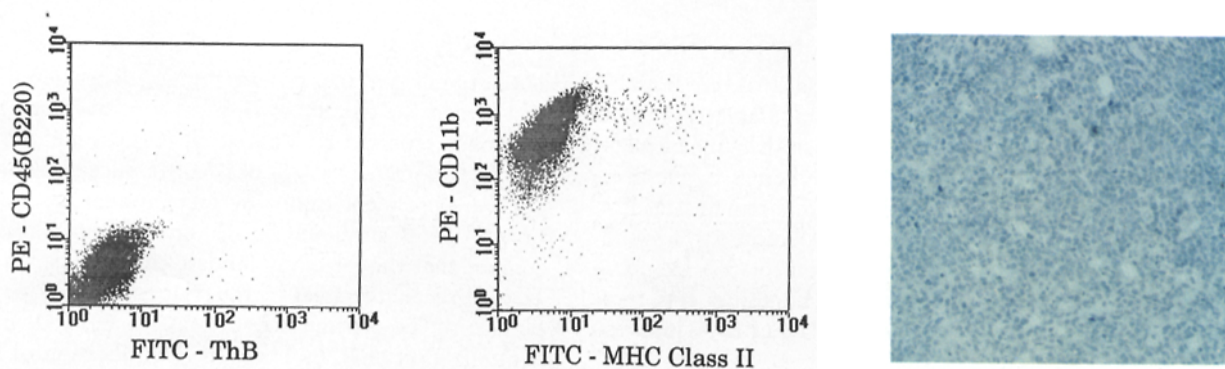
A**B**

Figure 1. Flow cytometric and immunohistologic analyses of J3V1-induced tumors. (A) Typical plasma cell tumor phenotype. (B) Typical myeloid tumor phenotype. Flow cytometric analyses were performed on tumor cells recovered from ascites fluid of tumor-bearing mice. Immunohistochemistry was performed using dioxigenin-labeled L chain riboprobes specific for murine C κ and C λ . Hybridized probe was visualized with horseradish peroxidase-labeled sheep antidioxigenin Fab fragments and 3,3' diaminobenzidine. Plasma cell tumors expressing either IgL κ or λ are stained brown, whereas myeloid tumor cells exhibit only the hematoxylin counterstain.

(data not shown). Analysis of tumors arising in +/+ and +/- mice (Table 1) indicates that plasma cell tumors develop at incidences of 34 and 38%, respectively. These frequencies are comparable to the 50% observed among similarly treated BALB/c mice. Myeloid tumors also develop in +/+, +/-, and BALB/c mice at comparable incidences of 32, 33, and 30%, respectively. Chi square analysis indicates that there are no significant differences in the occurrence ($\chi^2 = 1.73$, $P > 0.35$) or latency ($\chi^2 = 2.09$, $P > 0.42$) of myeloid and plasma cell tumors in these animals. In contrast, mice homozygous for the IL-6-null allele (-/-) are completely resistant to *myc/raf* plasma cell tumorigenesis, thereby defining an essential role for IL-6 in the development of these tumors in vivo. These results do not preclude the possibility that other oncogenes or cellular mutations may induce IL-6-independent plasma cell tumors under similar conditions.

It remains unclear at which point in tumor development IL-6 exerts its effects. In keeping with its role in vitro, IL-6 may function as a late-acting pristane/granuloma-derived progression factor that induces the proliferation or facilitates survival of transformed plasma cells. Without this cytokine

such cells fail to expand and/or survive, thus precluding the occurrence of overt tumor. Alternatively, IL-6 may act much earlier in tumor development by promoting the appearance of appropriate target cells in which transforming oncogenes may exert their effects. It is also possible that IL-6 may be required at multiple steps in this pathway. Regardless of where the IL-6 requirement is manifested in tumorigenesis, it may not correspond precisely to an analogous point in the generation of normal plasma cells. This suggestion follows the observation that IL-6-deficient mice contain normal numbers of IgM-secreting plasma cells and can produce antigen-specific IgM in response to challenge with foreign antigen (31, 37). The existence of IL-6-independent plasma cells in -/- mice suggests that the failure to generate IgM-secreting plasma cell tumors is due to a tumor-specific requirement for signal(s) unique to the IL-6/IL-6R transduction pathway.

The resistance of IL-6-deficient mice to plasma cell tumor induction appears to be lineage specific, since myeloid tumors are detected in -/- mice with an incidence comparable to that seen among +/+ and +/- animals. These results indicate that myeloid tumorigenesis proceeds via an IL-6-inde-

Table 1. Incidence and Phenotype of J3V1-induced Tumors in Normal and IL6-deficient Mice

Genotype	Number of mice*	Tumor latency [†]	Tumor phenotypes [‡]			
			Plasma cell tumor	B cell lymphoma	T cell lymphoma	Myeloid
BALB/c	10	83 ± 5	50 (5)	0 (0)	0 (0)	30 (3)
+ / +	47	88 ± 5	34 (16)	2 (1)	0 (0)	32 (15)
+ / -	21	97 ± 5	38 (8)	0 (0)	0 (0)	33 (7)
- / -	35	98 ± 7	0 (0)	3 (1)	6 (2)	37 (13)

* Number of mice receiving intraperitoneal injection of pristane/J3V1. Three mice in the + / + and - / - groups died during the experiment without indication of tumor development.

† Calculated as the number of days between virus injection and appearance of tumor ± SEM.

‡ Percentage of tumor-bearing mice of indicated hematopoietic lineage (number of mice developing tumor).

pendent pathway. Similarly, the appearance of rare B and T cell lymphomas in + / + and - / - groups (Table 1) suggests that they too may occasionally arise in an IL-6-independent fashion. The relationship of these lymphomas to the more common myeloid and plasma cell tumors remains unclear, but they have been seen sporadically in all mouse strains subjected to the pristane/J3V1 induction protocol (34), including plasmacytoma-resistant DBA/2 mice, suggesting that these lymphomas may represent a distinct class of IL-6-independent tumors.

The failure of IL-6-deficient mice to develop plasma cell tumors is somewhat surprising in light of the apparent functional redundancy inherent in many cytokine pathways within the immune system. Recent reports have suggested that oncostatin M, leukemia inhibitory factor, IL-11, and ciliary neurotropic factor share many of the biological functions associated with IL-6 (for review see reference 4). Most relevant to the current study is the ability of these factors to stimulate the proliferation of several IL-6-dependent human myeloma cell lines through use of cytokine-specific receptors and the common gp130 signal-transducing molecule (4, 29, 30). Our results indicate that these functionally redundant cytokines cannot replace the unique and requisite signals imparted by IL-6 during the genesis of murine plasma cell tumors. However, it remains unclear if these cytokines are capable of supporting the in vivo growth of an established IL-6-dependent murine plasmacytoma in a manner analogous to that described for their support of human myeloma lines in vitro. To address this question, we challenged IL-6 + / + and - / - mice with the IL-6-dependent murine plasmacytoma T1165. As shown in Table 2, T1165 grew only in those mice that were both IL-6 competent and that had been treated with a single injection of pristane to condition the peritoneal cavity. IL-6-deficient mice (- / -) failed to support the in vivo growth

of this tumor even after pristane priming. These results confirm and extend the efficacy of IL-6 deprivation-based therapies originally reported by Vink et al. (17), in which they successfully protected ~50% of BALB/c mice challenged with an IL-6-dependent tumor by pretreatment with anti-IL-6 or anti-IL-6R antibody. By analogy, the current results also suggest that the relatively modest clinical benefits of anti-IL-6-mediated therapies for the treatment of plasma cell neoplasia may be attributable, at least in part, to inefficient neutralization of IL-6. Thus, the identification of IL-6 as a critical in vivo growth factor for the emergence and subsequent propagation of plasma cell tumors provides experimental evidence to support continued efforts to develop more efficient IL-6 deprivation-based therapies for the treatment of human B cell malignancies.

Table 2. Growth of IL-6-dependent Plasmacytoma T1165 in Normal and IL-6-deficient Mice

Host genotype	Pristane*	In vivo survival of IL-6-dependent T1165 cells [†]
BALB/c	-	0/4
BALB/c	+	4/4
+ / +	-	0/4
+ / +	+	3/3
- / -	-	0/4
- / -	+	0/4

* 0.5 ml pristane administered intraperitoneally 2 d before tumor transfer.

† Number of mice in which tumor grew/total number injected with 5×10^6 T1165 cells.

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